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Description

The description relates to a method of producing IFN beta in an animal host cell. The IFN beta isolated with high yield from the cell supernatant has a specific activity of about 3.8×10^8 IU/mg protein. Biological and immunological tests show that the isolated IFN-beta is substantially identical with natural IFN-beta. The IFN-beta produced by the method described is 95% glycosylated, and the structure and sequence of glycosylation is substantially similar to but not identical with that of natural IFN-beta.

Interferons are a group of antivirally active polypeptides formed through contact with exogenous inductors (e.g. viruses, nucleic acids or certain antigens) of the cells in question. One sub-group, beta-interferons (IFN-beta), are formed mainly by fibroblasts, Havell et al (1972) and Stewart II (1979). At present there are two known species of beta-interferons. Their immunological properties are similar, so that monoclonal neutralising antibodies can be isolated which activate both interferons similarly - see Zilberstein et al (1985). On the other hand there is no cross-hybridisation between IFN-beta-2 mRNA and samples of IFN-beta-1 cDNA in RNA gel blot hybridisation experiments or vice versa, Sehgal et al (1980).

IFN-beta-1 from human diploid fibroblasts (FS4), hereinafter called IFN-beta, has been in clinical use for some time. As early as 1983 it was licensed by the German Federal Office of Health for treatment of severe life-threatening virus infections. Owing to its efficiency and the absence of other wide-spectrum virostatics, it has become the preferred substance in many cases.

However, clinical use of the drug is greatly restricted by the high price, since production on the basis of normal non-transformed diploid fibroblasts requires use of rare, expensive raw materials and of cost-intensive cell substrates, and since there are narrow limits to the extent to which the processing operations can be rationalised.

This situation very quickly stimulated a search for alternative production methods, and was one of the most important motives for the development of modern genetic-engineering methods in general. Inclusion of the gene for human IFN-beta in heterologous host cell systems was obviously the only true alternative to conventional optimisation processes, since their prospects of success appeared greatly restricted by physiological barriers.

Out of the three main available groups of host cell systems, i.e. procaryotes, lower and higher eucaryotes, the general preference was initially for the bacterial host system Escherichia coli (E.coli)

on the basis of the prior art and the extremely low production costs.

In spite of the high expectations regarding this production system, it has not hitherto been possible to process the large amounts of crude IFN-beta obtainable by this method on an industrial scale, Taniguchi et al (1980) and Goeddel (1980). This difficulty is mainly due to the fact that the raw material is present in the form of denatured inclusion bodies in the host cell. This enables the constituents of the host cell to be efficiently separated, but hitherto the yield of clinically useful material has been very low owing to the problems of subsequent processing (difficult solubility, occurrence of incorrect sulphur-bridge bonds). There is a partial lack of activity probably due to conformation changes as a result of denaturing or incorrectly formed intrachenar sulphur bridges, Lawn et al (1981), and additionally the product, in contrast to the naturally occurring form, is not glycosylated.

In this situation it has become increasingly necessary to push forward with the development of eucaryotic expression systems whereby even heterologous gene products of more complex structure can be produced in substantially authentic form by correct "processing". Systems of this kind have been described by a number of authors over the years, Reyes et al (1982), Mitrani-Rosenbaum et al (1983), Smith et al (1983), McCormick et al (1984),

Chernajovsky et al (1984), Fukunaga et al (1984), Page et al (1985).

Basic construction

We shall now describe the construction of a new BIC cell line by genetic-engineering methods whereby large quantities, compared with conventional methods, of a native product substantially identical with the natural substance can be produced at substantially lower cost.

Host cell line

The chosen host cell line was the DHFR$^-$ mutant of the permanent CHO (Chinese hamster ovary) line, described by Urlaub et al (1980).

Vectors

The interferon gene

The starting material was the plasmid vector pBR 13, which contains the genomic IFN-beta gene. A defined portion of this genomic DNA was shortened by the methods described by Maniatis et al (1982) and recombined with an already-known vector (pSVd2-3).

Promoter

Another important step was the selection of a suitable promoter for regulation of expression. Notwithstanding the possibility of using inducible promoters, Hauser et al (1982) and Brinster et al (1982), we preferred the strong constitutive promoter of SV40 described by Mosthaf et al (1985), which also includes an enhancer region. We came to this decision after our own discouraging experience with induction-dependent production processes (negative feedback, cytotoxicity) on the one hand and the possibilities offered by permanent fermentation processes even for obligate adherent cell cultures on the other hand. The construct, described in further detail hereinafter, was called pSVIFNAsu.

Selection

For the purpose of selection and amplification in the direction towards a high-expressing cell line, the DHFR gene was separately introduced into a second plasmid pAdD26SV(A)-3. This was a means of selection on the basis of IFN-beta expression, owing to the interaction between the individual adenopromoter and the enhancer region of the SV40 promoter (see above). In the present construct, as expected, the common transfected DNA was incorporated in close proximity.

Transfection

The above expression vector, which contains regulating sequences and the structure gene, and the selection plasmid, which repairs the deficiency in the cell line, were transfected together in an experimentally determined optimum mixing ratio in accordance with a calcium phosphate precipitation method.

Amplification, cloning and cell bank

Interferon-expressing clones from the transfection batch were amplified by a method first described by Kaufman et al (1985) over a number of stages of methotrexate concentration without intermediate selection on high-producing colonies, and were then cloned and after serial passages acquired a sufficient number of cells, which were stored at a stem cell bank in liquid nitrogen in accordance with conventional methods of conservation.

Results

Production of the raw material

The cell line obtained by the method described and given the laboratory designation BIC 8622 (BIC, ECACC Entry No. 87040301) secreted after confluence in conventional cell culture medium (modified Eagle's MEM with Earle's salts) supplemented with 1.5% NCS or FCS in stationary culture in multitrays

(NUNC) e.g. in the fermenter on micro-supports (Cytodex III, Pharmacia), consisting of between 0.4 and 0.6 x 10^9 international units of IFN-beta per day in a litre of culture supernatant.

Concentration

Concentration was via adsorption on a sulphopropyl cation exchanger and subsequent immune sorption on anti-IFN-beta sepharose (Celltech, Slough). Since this immune sorption matrix contains monoclonal antibodies against native fibroblast interferon, adsorption and desorption under the conditions optimised by the manufacturer for the immune binding reaction, is an indication of the identity of the synthetic molecule, which could be quantified by an ELISA developed by using the same antibodies. This was followed by a purification step consisting of FPLC gel filtration for removing low-molecular and high-molecular attendant materials.

Characterisation

Biological effect:

The bioassay developed for determination of natural IFN-beta from FS-4 cells, using the antiviral activity by measuring the inhibition of the cytopathic effect of murine encephalomyocarditis virus (EMCV) on an indicator cell (FS-4), modified after Havell et al (1972), is also usable without limitation for determination of IFN-beta from BIC

cells. The specific activity obtained by this test and by protein determination after Lowry et al (1951) is 2-3 x 10^6 IU which, within the limits of accuracy of measurement, corresponds to the data obtained for FS-4 interferon.

Immunological characterisation:

In addition to immune affinity chromatography used for concentrating IFN-beta from BIC, a newly developed ELISA and immune blotting techniques showed substantial agreement in molecular properties between natural and recombinant IFM-beta. The antibodies used were monoclonal mouse hybridomas (MAK BO-2, Celltech) and polyclonal IgG from the goat (Rega Institute, Leuwen).

Protein chemical characterisation:

The data obtained by immunological methods were supplemented by amino acid analysis and sequencing of 15 N-terminal amino acids. No differences could be shown between natural, Knight et al (1980) and recombinant IFN-beta. Investigation of the carbohydrate component yielded substantially uniform glycosylation for the recombinant IFN-beta, whereas the natural IFN-beta carbohydrate was heterogeneous.

Experimental part

Materials

E.coli K12 DH 1 (DSM)4079	
CHO DUK DHFR$^-$BF (ECACC Entry No. 87041401)	This cell line originated from mutagenesis and selection for absence of dihydrofolate reductase from the line CHO-K1 (ATCC CCL 61), Urlaub et al (1980).
pBR 13: (DSM 4074P)	This plasmid is a derivative of the cloning plasmid pBR 325, Bolivar et al (1977), having a single EcoRI interface into which a 1.83 kb long DNA fragment was inserted. This fragment is contained in the cosmid pCos IFN-beta (Gross et al 1981), which itself is part of a cosmid bank of human placenta DNA.
PSVd2-3: (DSM 4075P)	This plasmid is a derivative of the cloning vector pAT 153 which contains sequences from the eucaryotic DNA virus SV 40, Fiers et al. (1978). The starting plasmid for constructing the

corresponding vector plasmids was a construction for expressing the mouse dihydrofolate reductase gene, Subramani et al. (1981). The modification of the expression vector pSV used was produced by Dr D Huylebroeck and is substantially described in the work by Fransen et al. (1985).

pAdD26SV(A)-3: (DSM 4076P) A plasmid derivative originating from deletion of a 1.1 kb long DNA fragment from the cloning vector pBR 322, Lusky et al. (1981). The plasmid also contains the transcription initiation signal (adenovirus major late promoter) from the eucaryotic DNA virus adeno 2, together with the cDNA of the mouse dihydrofolate reductase gene, the poly-adenylation signal and a 200 bp long fragment with the origin of replication from SV 40, (Kaufman et al. 1982a).

The expression system

The expression system used is based on a line of egg stem cells from the Chinese hamster (CHO, ATCC CCL 61 CHO-K1) made deficient in the dihydrofolate reductase enzyme by mutagenesis, Urlaub et al. (1980). By incubation of a calcium phosphate precipitate of DNA with these cells it is possible, with very low frequency, to obtain absorption of the DNA in the cells, Graham et al. (1973). The DNA thus introduced into the cells is usually combined and incorporated in the genome of the cell in question, where it is propagated or changed exclusively during cellular or DNA replication or during the recombination processes occurring in the cell genome, i.e. behaves like an integral natural component of the cellular genome. In the special case, an expression plasmid for expressing the human IFN beta gene is incorporated in the CHO cells together with a plasmid available in the mixture for expression of the cDNA dihydrofolate reductase gene from the mouse. The status of the expression plasmid can be determined by the so-called Southern Blot technique. To this end the cellular DNA is cleaved with restriction endonucleases and separated on an agarose gel in an electric field. The DNA fragments are then bonded to a nylon filter membrane and DNA is detected by DNA-DNA hybridisation with radioactively labelled plasmid.

Isolation of the interferon beta gene

The starting plasmid for isolation of DNA restriction fragments with the IFN beta gene was the plasmid pBR 13. In order to produce this plasmid, first the mRNA coding for IFN beta was transferred from FS 4-fibroblasts to cDNA and a cosmid pCOSIFN beta containing the IFN beta gene was isolated from a gene library (human placenta) by hybridisation with complementary genomic DNA. By cleaving the plasmid pBR 13 with the restriction endonuclease Eco RI, the fragment containing the IFN beta gene was first isolated from pCosIFNbeta and then was isolated, followed by further cleavage with the restriction endonucleases Hinc II or Nco I and Hind III, yielding smaller DNA pieces with the IFN-beta gene.

Construction of the expression plasmids

The plasmid for expression of the human IFN beta was constructed by using an expression vector called pSVd2-3. The functional components of this plasma ensure autonomous DNA replication in E.coli and selection of the E.coli cells by ampicillin (origin of replication of the transfer-negative plasmid pAT 153 and of the β-lactamase gene). The components determined for operation in eucaryotic cells consist of the promoter-enhancer region of the early genes of the virus SV 40 for initiation of transcription of the downstream genes and of the polyadenylation signal of the SV 40 virus for attaching a polyA

sequence to the transcribed mRNA if a signal sequence of this kind is lacking in the gene used. Between the transcription initiation signal and the polyadenylation sequence there are number of synthetically produced recognition and cleavage sequences for restriction endonucleases for insertion of DNA for expression. The cleavage site of the nuclease Xba I was chosen for cloning the human IFN-beta DNA in this vector. To this end the vector was cleaved with Xba I. An Eco RI fragment with IFN beta gene was first isolated from the plasmid pBR 13, after which an Ncol - Hind III sub-fragment was isolated therefrom with IFN-beta gene. The overhanging single-stranded DNA ends produced by cleavage with the restriction endonucleases were filled by incubation with nucleoside triphosphates and the DNA polymer salt from E. coli. Synthetically produced polynucleotides containing the expression sequence of the restriction endonuclease Xba I were then enzymatically attached to the resulting smooth DNA ends of the fragment. Excess oligonucleotides were removed by incubation with the nuclease Xba I, which simultaneously resulted in overhanging single-stranded ends which were complementary with those of the vector DNA. The vector DNA and the IFN-beta DNA were enzymatically fitted together and introduced into E. coli cells. The cells containing IFN-beta DNA were identified by DNA-DNA hybridisation and used for purifying larger amounts of plasmid DNA. The plasmid DNA was characterised by cleavage with various restriction endonucleases and each plasmid

was selected for further processing and called pSV IFN Nco (DSM 4077 P), which conformed to expectations. The plasmid pSV IFN Nco was then partially cleaved with the enzyme Xba and the fragments were separated by gel electrophoresis. The bands corresponding to the linear plasmid were dyed with the fluorescence dye ethidium bromide and made visible in UV light and cut out and the DNA was isolated. The isolated DNA was then incubated with the nuclease Asu II and then enzymatically closed in a ring. This procedure resulted in deletion of an Xba I-Asu II fragment from the original expression plasmid pSV IFN Nco. The resulting expression plasmid was called pSV IFN Asu (DSN 4078 P) and used for transfection of a CHO cell line deficient in dihydrofolate reductase. Cells which had been transfected with the expression plasmid pSV IFN Asu and had incorporated their DNA in the cell genome were made to form an mRNA consisting of a 60-nucleotide portion of SV 40-specific sequences and practically the authentic IFN beta mRNA attached thereto. This mRNA was transferred by the protein synthesis apparatus of the cells into an IFN-beta protein which, in its amino-terminal region and its amino acid composition, corresponded to the natural IFN-beta protein from human fibroblast cells and was glycocylated, in contrast to the proteins produced in E. coli.

The DNA species and the manner of construction of the expression plasmid pSV IFN Asu can be used to derive the primary structure of the mRNA formed in

eucaryotic cells which had incorporated this plasmid in their genome. Fig. 2 shows the structure of the expression plasmid with a detailed print-out of the relative cleavage sites. Transfer of the nucleotide sequence into an amino acid sequence by the genetic code yields the primary structure of the authentic IFN beta from human cells, Tavernier et al. (1984).

Transfection

The expression vector DNA was introduced into the CHO cell line substantially by a method described by Graham et al. (1973) and modified by Wigler (1979). Basically in this method, the cells are supplied with DNA precipitated from a solution containing CaPO4 coprecipitate. The DNA is then absorbed by the cells, probably by phagocytosis, and mainly lined up by unexplained mechanisms and incorporated in the cellular genome. This process is called transfection of eucaryotic cells with DNA. By combined precipitation of a vector for expression of the enzyme dihydrofolate reductase with the actual expression vector, when using dihydrofolate-deficient CHO cells, the transfection process can be used to select those cells which have absorbed DNA and incorporated it in their genome. This is done by cultivating the cells in a cell culture medium which lacks the components for nucleic acid synthesis.

Amplification and selection

The culture supernatants of the thus-transfected and selected cells were tested for beta-interferon activity. The positive clones in this test were subjected to a process for selectively multiplying the DNA incorporated in the cells and increasing the number of copies of the interferon gene in order to increase the production of beta-interferon. This is possible in principle by the gene-dose effect. The method used for this purpose involves selection of the beta interferon-producing cells in a culture medium containing the enzyme inhibitor (+) amethopterin. By means of this inhibitor, the enzyme dihydrofolate reductase, whose gene has been introduced into the CHO cells by transfection, is inhibited in dependence on concentration. As a result of this inhibition, those cells which have increased the dihydrofolate reductase gene dose by multiplication of the corresponding DNA have a growth advantage. Since these DNA multiplication processes usually involve larger portions and the transfected DNA species are usually incorporated close together in the genome of the cells, the dose of the beta-interferon gene is simultaneously increased. Stepwise increase of the (+) amethopterin concentration in the culture medium and intermediate selection and expansion phases result in production of a cell mixture which delivers large amounts of beta interferon to the culture medium. This mixture, by dilute sowing of the cells in the culture vessels and isolation of the cell colonies

by means of metal cylinders, yielded pure lines of cells which likewise deliver large quantities of beta-interferon to the culture medium.

A producer cell library (= production cell library) was produced from these clones, called "BIC" cells, in accordance with the relevant guidelines, and portions of these cells were deposited in the "Public Health Laboratory Service, European Collection of Animal Cell Cultures (ECACC)", entry number 87040301.

Concentration

15 litres of culture supernatants with an average interferon content of 225 000 IU/ml were pooled by repeated harvesting in a 24-hour cycle from a confluent stationary culture of BIC taken from the production cell library and were together placed on a cation exchanger (sulphopropyl). 175 ml of eluate contained 3×10^9 IU, corresponding to an 83% yield. Subsequent immune sorption on BETA RESOLUTE R (Celltech) yielded an elution volume of 112 ml, containing 2.2×10^9 IU or 72% of the interferon with a specific activity of 3.8×10^8 IU/mg protein. Final gel filtration resulted in a total yield of 48%.

Analysis

Biological and immunological characterisation

Highly purified preparations of recombinant BIC beta-IFN and natural FS 4-beta IFN were tested for their interferon content in an antiviral bioassay. In parallel therewith, the protein content was determined after Lowry. The natural material had a specific activity of about $2.7 - 3 \times 10^8$ international units (IU) per milligram protein and the recombinant material had an activity of $3 - 4 \times 10^8$ IU/mg. These values correspond to the data known from the literature for natural interferon. The specific activity in the prior art cannot at present be determined more precisely, owing to the relatively high inaccuracy of the bioassay.

The starting material and the concentrated highly-purified fractions were also subjected to an enzyme-coupled immune test (Elisa) after the antiviral bioassay.

In this test the plastic surface of microtitre plates is coated with polyclonal goat anti-IFN beta antibodies. The sample under test is then diluted. The beta-IFN bonds to the immobilised antibodies. In the next step this complex is reacted with a solution of monoclonal mouse anti-IFN beta antibodies. The resulting total complex is incubated with anti-mouse IgG antibodies previously conjugated with horseradish peroxidase. After

removal of the non-bonded antibodies, the quantity of bonded peroxidase and consequently the quantity of bonded beta-IFN can be determined by a colour reaction.

This test was validated for natural beta-IFN obtained from FS 4 fibroblasts. To this end dilution series of samples having a known interferon content were tested together with portions of the international beta-IFN standard (NIH, G-023-902-527). In all cases there was a close correlation between the bioassay and the ELISA.

The concentrated, highly purified IFN preparations obtained from the culture supernatants of BIC cells uniformly corresponded in the ELISA to the values obtained in parallel in the antiviral bioassay. Aliquot portions of the international standard or of preparations calibrated therewith were used for standardisation in all cases. This therefore shows that the antigenic properties relevant to the Elisa were also identical between natural and recombinant beta-IFN.

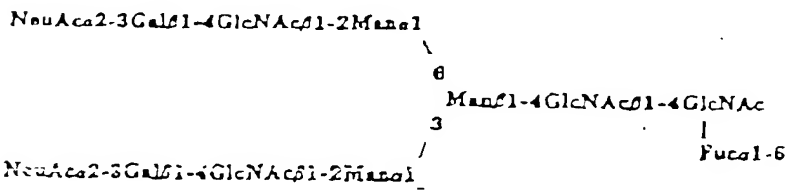
Sequence/amino acid composition

The recombinant IFN-beta produced and purified by the method described was analysed with regard to its amino acid composition and its amino-terminal sequence.

Partial sequencing up to the 15th amino acid was effected by Edman decomposition in an automatic gas phase sequencer (Messrs Applied Biosystems, Type 470 A). The resulting phenyl hydantoin derivatives were separated on a PTH-C80 matrix and then detected. The result agrees with the data published by Lawn et al (1981), Ohno et al (1981) and Derynck et al (1980). Analysis of the amino acid composition, wherein the PTH amino acids were subjected to the same methods of separation after hydrolysis, show that the values obtained by Knight et al (1980) for FS-4 were also true for the IFN-beta from CHO.

Glycosylation

The polypeptide obtained after deglycosylation or after inhibition of glycosylation with tunicamycin had the same electrophoretic properties under denaturing and non-denaturing conditions as the similarly obtained IFN beta from FS-4. The oligosaccharides liberated by glycopeptidase F after sequential decomposition by exoglycosidase, were subjected to methylation analysis and FAB mass spectrometry. It was shown that 95 ± 5% of the carbohydrate side chains were biantennary complex type and had the following structure:



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Claims

1. Recombinant cell line BIC 8622 (ECACC 87040301).

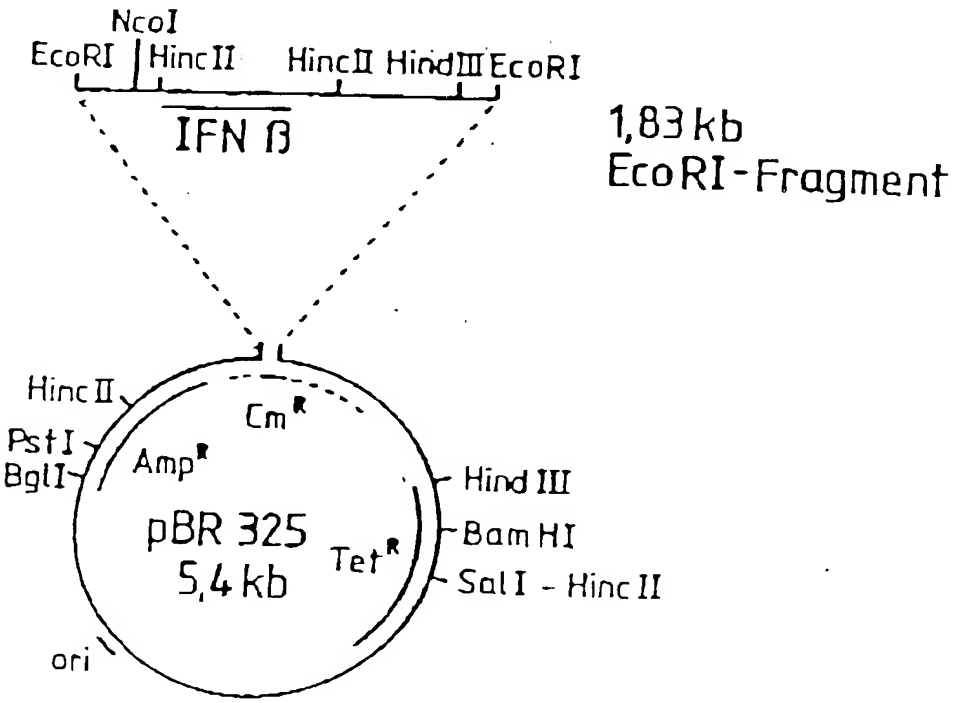
2. Process for the constitutive preparation of human IFN-β1, wherein a recombinant cell line according to claim 1 is cultured and the IFN-β1 is isolated from the cell supernatant.

3. Process according to claim 2, further comprising the use of the human IFN-β1 obtained for the preparation of a medicament.

4. Process according to claim 3 for the preparation of an agent for the treatment of virus infections.

Fig. 1

pBR 13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EcoRI
HincII
HincII
HindIII
EcoRI
IFN β
1,83 kb
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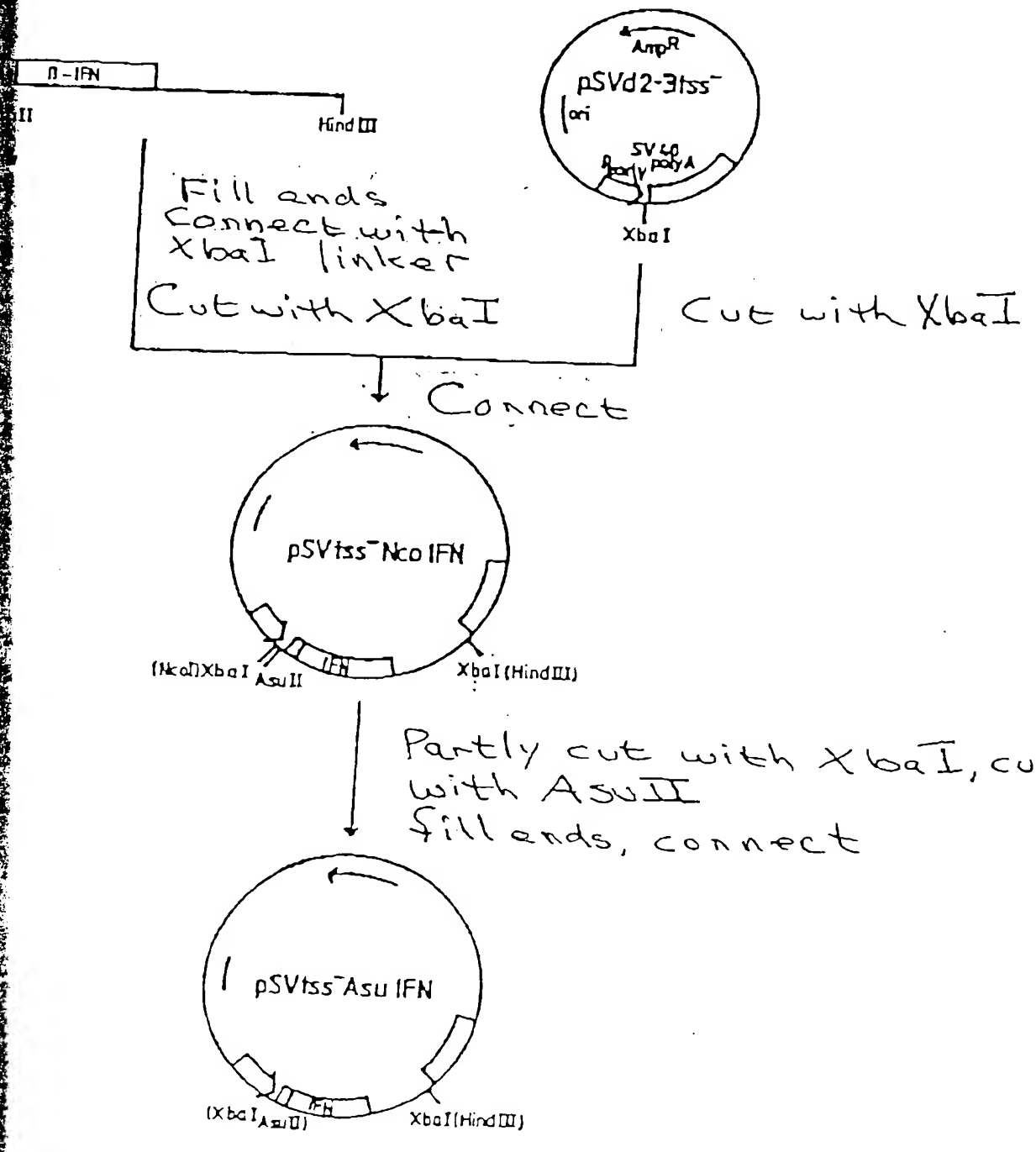
HincII
PstI
BglI
Amp^R
Cm^R
pBR 325
5,4 kb
Tet^R
Hind III
Bam HI
Sal I - Hinc II
ori

Fig. 11


β-IFN
Hind III
pSVd2-3tss⁻
AmpR
ori
SV40 poly A
XbaI
Fill ands
connect with
XbaI linker
Cut with XbaI
Cut with XbaI
Connect
pSVtss⁻Nco IFN
(NcoI)XbaI
AsuII
XbaI (HindIII)
Partly cut with XbaI, cut
with AsuII
fill ands, connect
pSVtss⁻Asu IFN
(XbaI AsuII)
XbaI (HindIII)